Differential mRNA expression levels and gene sequences of a putative carboxylesterase-like enzyme from two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae)

Yu-Cheng Zhu, Alan K. Dowdy, James E. Baker [*]

Grain Marketing and Production Research Center, ARS-USDA, 1515 College Avenue, Manhattan, KS 66502, USA

Abstract

Carboxylesterase-like enzyme cDNAs have been cloned and sequenced from malathion-resistant and susceptible strains of the parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae). The cDNAs consist of 1963 nucleotides including a 35 bp untranslated 5′-end, a 1596 bp open reading frame, and a 332 bp untranslated 3′-end. The open reading frame encodes 532 amino acid residues. The predicted protein sequence from these cDNAs includes 2 potential N-glycosylation sites, a carboxylesterase type-B serine active site FGGDSENVTIFGE**S**AG, and conserved residues Ser^{187}, Glu^{317}, and His^{432} to function as the catalytic triad. The predicted carboxylesterase-like enzyme sequence is most similar to that of the carboxylesterase from the peach-potato aphid, *Myzus persicae* with 45% sequence identity. Alignment of the parasitoid carboxylesterase-like enzyme cDNAs revealed that there are two nucleotide differences in the open reading frame between the parasitoid strains, including a silent mutation and a point mutation that presumably causes a gene product difference. A nucleotide thymine at position 658 in the susceptible strain cDNA is replaced by a guanine in the resistant strain cDNA. This substitution leads to an amino acid change from tryptophan (Trp^{220}) in the susceptible strain to glycine (Gly^{220}) in the resistant strain. This substitution is genetically linked to resistance but it is not known how or if this amino acid substitution affects detoxification of malathion. Northern blot analyses demonstrated that expression level of the carboxylesterase-like enzyme mRNA in adult *A. calandrae* is approximately 30-fold higher in the resistant strain relative to that in the susceptible strain. Southern analysis indicated that *Pst I* or *Eco RI* restriction sites are different in the two strains. Both a modified gene structure and an increase in expression of carboxylesterase may be responsible for the high level of resistance found in this beneficial wasp. Published by Elsevier Science Ltd.

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1. Introduction

Malathion resistance in the parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) is associated with a 10 to 30-fold increased activity of a malathion-specific carboxylesterase (MCE) (Baker et al., 1998). There is no evidence that other detoxification systems such as general esterase activity, phosphotriesterase, glutathione S-transferase, monooxygenase, or altered acetylcholinesterase contribute significantly to the

resistance. MCE activity in *A. calandrae* is cytosolic with optimum activity at pH 7 and with p*I* 5.2. MCE can be recovered from native polyacrylamide (PAGE) gels, however, the activity co-elutes with the main peak of activity against α-naphthyl acetate.

We are currently attempting to purify the MCE but are also interested in characterizing the molecular basis of the resistance at the gene level. In this paper we describe the full sequence of carboxylesterase-like enzyme cDNA clones from both malathion-resistant and susceptible strains of *A. calandrae* and document a point mutation that results in an amino acid sequence change, Trp^{220} to Gly^{220}, in a carboxylesterase in the resistant strain. This point mutation has been shown by PCR amplification of specific allele (PASA) to be genetically

* Corresponding author. Tel.: +1-785-776-2785; fax: +1-785-537-5584
E-mail address: baker@usgmrl.ksu.edu (J.E. Baker)

linked to resistance (Zhu et al., 1999). In addition, we demonstrate that expression levels of carboxylesterase-like enzyme mRNA are significantly higher in the resistant strain compared with that in the susceptible strain.

2. Materials and methods

2.1. A. calandrae strains

A susceptible laboratory strain of *A. calandrae* has been maintained in culture for more than 20 years. A malathion-resistant strain was collected from a farm near Bamberg, SC, in 1992 and subsequently maintained without selection pressure in the laboratory. For this study, the resistant strain was selected one time with malathion (Baker et al., 1998). Both strains are reared on immature rice weevils, *Sitophilus oryzae* (L.), infesting wheat at 27°C and 55–65% RH.

2.2. RNA and poly(A) RNA purification

Two grams of fresh adults from each strain were ground in liquid nitrogen. Total RNA was extracted with guanidine thiocyanate denaturing solution and precipitated with isopropanol (Titus, 1991). The poly(A) RNA was isolated from the total RNA by chromatography using an oligo(dT)-cellulose column (Gibco BRL Life-Technologies, Gaithersburg, MD).

2.3. Construction of cDNA libraries

Double stranded cDNA was synthesized using 5 μg of poly(A) RNA as a template (ZAP–cDNA synthesis kit, Stratagene, La Jolla, CA), directionally cloned into a UniZAP XR λ phage vector phage (Stratagene), and packaged using the ZAP–cDNA Gigapack II Gold packaging extract (Stratagene). Approximately 1.3 and 1.0 million recombinants were obtained for susceptible and resistant parasitoid cDNA libraries, respectively.

2.4. Development of probes

Lambda DNA of an amplified cDNA library from the resistant strain was prepared using phage precipitation and phenol/chloroform extraction procedures after RNase A and DNase I digestions (Titus, 1991). Carboxylesterase cDNA fragments were amplified using an oligonucleotide complementary to the T3 promotor in the vector as the forward primer and a reverse degenerate primer designed from a conserved region (FGESAGG) of carboxylesterases from mosquitoes and rats (Vaughan and Hemingway, 1995; Yan et al., 1994). PCR products were separated on a low melting point agarose gel. DNA fragments of the expected size (~700 bp) were sliced from the gel and extracted using a DNA purification system (Promega, Madison, WI). The isolated DNA fragments were ligated into a pGEM–T vector (Promega) overnight at 14°C. *Escherichia coli* JM109 competent cells (Promega) were transformed and plated on LB/ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. A PCR-confirmed colony was used to inoculate a 50 ml LB/ampicillin culture. Plasmid DNA was extracted using a Qiagen Plasmid Midi kit (Qiagen, Santa Clarita, CA). The DNA inserts were sequenced using a thermal cycling and silver staining sequencing kit (Promega) and an automated sequencer (Applied Biosystem Model 393A). Carboxylesterase-like fragments were confirmed by Blastx similarity searching of the GenBank database.

2.5. cDNA library screening

The cDNA libraries were plated to a density of ~130 plaque forming units per square centimeter, and plaques were transferred to nylon membranes (MSI, Westboro, MA). The membranes were hybridized at 55°C (LabLogix DNA/RNA Hybridization Kit, LabLogix Inc. Belmont, CA) with a PCR-amplified carboxylesterase-like cDNA probe labeled with $\alpha-^{32}$P-dCTP (Amersham, Arlington Heights, IL). Membranes were washed at 55°C for 1 h with three changes of 0.2×SSC (sodium chloride/sodium citrate)/0.1% SDS and then exposed to X-ray films. Positive clones from the first library screen were subjected to PCR amplification and analyzed for the presence of an insert fragment of the predicted size (~700 bp when amplified by T3 and degenerate priming, and ~2 kb when amplified by T3 and T7 priming). Positive clones after PCR were plated to a density from which a single plaque could be readily obtained without cross contamination. PCR amplification was repeated to locate a single plaque with an insert of the predicted size from each clone identified with the ^{32}P-labeled probe. These positive clones were subcloned in vivo into pBlue–Script SK($\pm$) phagemid (Stratagene). The cDNA inserts were sequenced by primer walking from both directions using an automated sequencer.

2.6. Cloning and sequencing of 5′-end of carboxylesterase cDNA

mRNA was purified from both parasitoid strains using PolyATtract mRNA Isolation System (Promega). cDNA ends were synthesized and amplified using 5′-RACE System (Gibco BRL, Life Technologies) with three gene specific primers used for reverse transcription and two subsequent semi-nested PCR amplifications. PCR fragments (~300 bp) were cloned, and sequences were obtained. 5′-RACE was repeated with another extraction of mRNA from both strains.

2.7. Sequence analysis

The Blastx program (Altschul et al., 1990; Gish and States, 1993) was used to search the National Center for Biotechnology Information Internet server non-redundant amino acid sequence database. The Wisconsin Sequence Analysis Package GCG Unix version 9.0 (Genetics Computer Group, Madison, WI) including Pileup, Gap, Distances and Growtree programs was used to analyze the similarity of esterase sequences (gap weight=4, gap length weight=1). Sequence analysis tools of the ExPASy Molecular Biology Server of Swiss Institute of Bioinformatics were used to process data of deduced protein sequences.

2.8. Carboxylesterase-like gene expression in susceptible and resistant strains

Carboxylesterase-like gene expression in adult wasps of both strains was analyzed by Northern blotting (Ausubel et al., 1994). Three µg of mRNA and 10 µg total RNA from each parasitoid strain was subjected to 1% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (MSI, Westboro, MA). A full-length carboxylesterase-like cDNA used as a probe was labeled with $\alpha-^{32}$P-dCTP. The target RNA was hybridized with the labeled probe at 55°C for 20 h. The nylon membrane was washed three times and exposed to X-ray film. To evaluate the quantity of RNA loaded in each lane, the carboxylesterase-like cDNA probe was removed from the nylon membrane using a boiling 0.5% SDS solution after the film was processed. The membrane was rehybridized with a $\alpha-^{32}$P-labeled ribosomal protein S3 cDNA from *Manduca sexta* as internal standard, which is highly conserved in sequence among bacteria, yeast, vertebrates, and invertebrates (Jiang et al., 1996). Autoradiographic data was analyzed using video densitometry program ITTI version 2.2 (Interactive Technologies International).

2.9. Southern blot analysis of genomic DNA from susceptible and resistant strains

Genomic DNA was extracted from adult parasitoids using an isolation buffer containing 100 mM Tris–HCl (pH 9), 1% SDS, and 100 mM EDTA. Southern analysis was used to search for esterase gene differences between the *A. calandrae* strains following procedures of Southern (1975), Wang et al. (1995) and Ausubel et al. (1994). Three double restriction enzyme digestions, *Pst I+EcoR I*, *EcoR I+Hind III*, *Hind III+Pst I*, and an undigested control were conducted for each insect strain. In each digestion, 5 µg of genomic DNA was digested with 25 units of each enzyme for 5 h at 37°C. Digested DNA was separated by electrophoresis on a 0.8% agarose gel, transferred onto a nylon membrane, and hybridized with a full-length carboxylesterase-like cDNA probe labeled with $\alpha-^{32}$P-dCTP at 55°C for 20 h. The nylon membrane was washed three times and exposed to X-ray film.

3. Results and discussion

3.1. cDNA sequences

A cDNA fragment from *A. calandrae* was amplified from the cDNA library extract using a T3 vector primer and a degenerate primer. The fragment was confirmed as carboxylesterase-like cDNA by Blastx searching of GenBank database, and was subsequently used as a probe for cDNA library hybridization. cDNA libraries from malathion-susceptible and resistant *A. calandrae* were screened using the probe labeled with $\alpha-^{32}$P-dCTP. Preliminary sequencing identified two clones from each cDNA library which were identical at their 3′-ends (~400 bp) and 5′-ends (~400 bp) and similar to the carboxylesterase-like cDNA sequence. One clone was selected from each library for sequencing and characterization.

Partial cDNAs of a putative carboxylesterase-like protein were obtained from the susceptible parasitoid library (1946 nucleotides) and the resistant parasitoid library (1771 nucleotides). Both cDNAs had identical 3′-ends and 18-base poly-A tails. A potential polyadenylation signal AATACA is located 36 bases upstream from the 3′-end of both cDNAs (Robin et al., 1996). A Blastx similarity search of the GenBank database and alignment of predicted carboxylesterase-like protein sequences with highly related esterase sequences from GenBank revealed that the parasitoid cDNAs were incomplete at their 5′-ends. To obtain the missing sequence, 5′-RACE was applied using 3 specific primers designed from cDNA sequences (Fig. 1). Approximately 300 bp at the 5′-ends of cDNAs from each parasitoid strain were cloned and sequenced. The 5′-end of cDNA from susceptible parasitoid was identical to that from the resistant strain. Complete cDNA from each strain contains 1963 nucleotides including a 35 bp untranslated 5′ sequence, an open reading frame of 1596 bp with the start codon ATG at positions 1–3 and the termination codon TAA at positions 1597–1599, and a 332 bp untranslated 3′-end (Fig. 1).

3.2. Deduced amino acid sequence

The open reading frame of the carboxylesterase-like cDNA from *A. calandrae* encodes a protein of 532 amino acid residues. The predicted protein sequence includes two potential N-linked glycosylation sites at positions 180 and 417 and a carboxylesterase type-B serine active site FGGDSENVTIFGESAG at positions 174–189 (Fig. 2). Analysis using SignalP (Nielsen et al.,

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                                    AGTTCTGCTGTGCACCTCGATTCATCACGTCTACA     -35

ATGGAACGACCAGAAGTGAAGACACTAAGCGGCCAAGTTCGAGGTCTGAAGCAAATTAGTGTTGAAGGCATAGGT     75
M   E   R   P   E   V   K   T   L   S   G   Q   V   R   G   L   K   Q   I   S   V   E   G   I   G     25

TTTTATGCGTTCAAGGGAATCCCTTACGCTAAGCCACCTGTTGGAGAACTCAGATTCAAGGATCCCGTCCCAATC    150
F   Y   A   F   K   G   I   P   Y   A   K   P   P   V   G   E   L   R   F   K   D   P   V   P   I     50

GAGCCATGGCAAGAAGTACGAGAAGCCACCGAATTCGGTCCAATGGCTGCGCAATTCGACGTGATATCGAAATTC    225
E   P   W   Q   E   V   R   E   A   T   E   F   G   P   M   A   A   Q   F   D   V   I   S   K   F     75

TCGGGCGGTTCGGACGACTGTTTGTACATAAACGTGTACACCAAGAAAATAAATTCAAACGTTAAACAGCCAGTC    300
S   G   G   S   D   D   C   L   Y   I   N   V   Y   T   K   K   I   N   S   N   V   K   Q   P   V    100

ATGTTTTACATTCACGGAGGTGGATTCATATTTGGTTCGGGCAACGACTTTTTCTACGGGCCTGACTTTTTGATG    375
M   F   Y   I   H   G   G   G   F   I   F   G   S   G   N   D   F   F   Y   G   P   D   F   L   M    125

AGAAAGGATATCGTGCTGGTCACGTTTAACTACAGATTGGGAGTGTTTGGATTTTTGAATTTGGAGCATGAAGTG    450
R   K   D   I   V   L   V   T   F   N   Y   R   L   G   V   F   G   F   L   N   L   E   H   E   V    150

GCGCCGGGAAATCAAGGTCTCAAGGATCAAGTAATGGCTCTGAAGTGGGTACGGGATAACATTGCGAATTTCGGT    525
A   P   G   N   Q   G   L   K   D   Q   V   M   A   L   K   W   V   R   D   N   I   A   N   F   G    175

GGTGATTCCGAAAACGTGACGATTTTCGGAGAAAGTGCCGGAGGTGCTTCCGTTCATTATTTGACTGTTTCGCCA    600
G   D   S   E   N   V   T   I   F   G   E   S   A   G   G   A   S   V   H   Y   L   T   V   S   P    200

                                                          G
TTGGCAAAAGGTCTCTTTCACAAGGCAATATCTCAAAGCGGAGTTTTTATGAACCCATGGGCATCAGTTTCTGGG    675
L   A   K   G   L   F   H   K   A   I   S   Q   S   G   V   F   M   N   P   W   A   S   V   S   G    225
                                                          G

GAACCAAGGAAAAAGGCTTACGAGCTCTGCGAGCTGCTGGGAAAGAAGACAACCGATCCAGTCGAAATCGTCAAA    750
E   P   R   K   K   A   Y   E   L   C   E   L   L   G   K   K   T   T   D   P   V   E   I   V   K    250

TTTCTGCGAACAGTCGACACCATGAAGCTGATTGAACATCAAGGAGAATTACAAATTCAAGAATTGCAAAAGAAG    825
F   L   R   T   V   D   T   M   K   L   I   E   H   Q   G   E   L   Q   I   Q   E   L   Q   K   K    275

TGTTTGAGCGCTTTCGTTCCGGGTGTCGACGACAAATCGCCGAACCCGTTCATGCCGTTCTCGCGCGAAGTAGCC    900
C   L   S   A   F   V   P   G   V   D   D   K   S   P   N   P   F   M   P   F   S   R   E   V   A    300

GTCGAGCAAGCCGCACACGTGCCTTACCTCATCGGATACAACGACAGGGAGGGAACACTGCTGTACAAAATTTTC    975
V   E   Q   A   A   H   V   P   Y   L   I   G   Y   N   D   R   E   G   T   L   L   Y   K   I   F    325

GAAAACGACGATTTCGAGAGCAAGAATCTGCGCTTCGAAGAGTTCATTCATCCAAACTTCGCGGAGACGTTGAAG   1050
E   N   D   D   F   E   S   K   N   L   R   F   E   E   F   I   H   P   N   F   A   E   T   L   K    350

                                                                   G
AGAAAGAAAATATCGCTGGAGGACTTGAAACGCATGTACTTCAAAAACAAGAAGATAAGCAAAGAGACTACAGGC   1125
R   K   K   I   S   L   E   D   L   K   R   M   Y   F   K   N   K   K   I   S   K   E   T   T   G    375

AAGTTCATCGATCTCTTCAGCGACATGTACTTCATTCAAGGAATTCATCAAGTCGCAAGGGTTCAAGCCGAGCGT   1200
K   F   I   D   L   F   S   D   M   Y   F   I   Q   G   I   H   Q   V   A   R   V   Q   A   E   R    400

AACTCCGCGCCTACGTACATGTACCAGTTCACGTACGATCAGGGGACCCAACTTTAGCAAAGGAATGTTCAGCATC   1275
N   S   A   P   T   Y   M   Y   Q   F   T   Y   D   Q   G   P   N   F   S   K   G   M   F   S   I    425

GACGAGCCTGGCTCGACGCACATGGACGAGCTGATCTACTTGTTTTCCATGAAGTTTCAGGAGACTCTTAACATG   1350
D   E   P   G   S   T   H   M   D   E   L   I   Y   L   F   S   M   K   F   Q   E   T   L   N   M    450

GAACCCATTGACAAGAAATCGCCTCATTTCAGAGTGATGGAACAGATGGTGGAGTTGTGGACCAACTTCGCGAAA   1425
E   P   I   D   K   K   S   P   H   F   R   V   M   E   Q   M   V   E   L   W   T   N   F   A   K    475

TACGGACGGCCGATACCCGCACCCACGGAACTTTTACCGGTACACTGGTTGCCAATGAATGACGGCACAGTACTT   1500
Y   G   R   P   I   P   A   P   T   E   L   L   P   V   H   W   L   P   M   N   D   G   T   V   L    500

CGCTACTTGAACATCGGCGAGGAGCTGCGTATGGAAAAAGTTTTAAATATCGAAGAACGTTACGATTACAAGCTT   1575
R   Y   L   N   I   G   E   E   L   R   M   E   K   V   L   N   I   E   E   R   Y   D   Y   K   L    525

ATATGCCATCGTGAAAAAGTCTAAAGAATCTGTGTTCTTTTTTTAAATTTCGAAAAGAATTGTACTACTTTTCTT   1650
I   C   H   R   E   K   V                                                                          532

TGCGTTTTTTTAACTTTTATTTAAATCATCATTCTTCGCGCAATTTTATGCACTGATAAAATGAAACGTCAAAATT   1725
TTTTATTATCGTTGATTGTTTCTTCGCTGTTTATTTTACTTTTAAACTGAAAAATTCTTGTTTATTCAATTGTTT   1800
TAACCTAATCTCTAGAATTTTACGATAAGTTTTATCAAATGTCAATCTTACCGATTCGTAAAGATAAAAATCATA   1875
GTAACTTGTAATAATACAACTTTAAAAAAAACGTCAAAAAAAAAAAAAAAAAAA                        1928
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Fig. 1. Nucleotide and deduced amino acid sequences of carboxylesterase-like cDNA isolated from *A. calandrae*. **ATG**=start codon; **TAA**=termination codon; **AATACA**=potential polyadenylation signal. Sequences used for 3 reverse primers in 5′-RACE are italic and bold. At positions 658 and 1122 in cDNA, bold letters indicate nucleotide differences between malathion-susceptible and resistant strains. Nucleotide substitutions from the resistant strain are located on the top of cDNA, and resulting different amino acid residue is located at the bottom of predicted protein sequence. The sequences have been deposited in the GenBank database (accession numbers AF064523 for the malathion-susceptible strain and AF064524 for the malathion-resistant strain of *A. calandrae*).

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AcCE   .............................MERPEVKTLS.....GQVRGLKQISVEGIGFYAFKGIPYAKPPVGELRFK
MpCE   ..........MKNTCGILLNLFLFIGCF.LTCSASNTPKVQVHSGEIAGGFEYTYNGRKIYSFLGIPYASPPVQNNRFK
CqAE   ..........................MDVEHPVVPT.....QYGPVRGVRKLAATGVDYYSFQRIPYVQPPVGELRFK
CtEB   .........................MSLENLTIQT.....KYGPVRGKRNVSLLGQEYVSFQGIPYARAPEGELRFK
DmAE   ..........................MSKESSL.ETCELTLPVGQIKGVKRLSLYDDPYFSFEKIPFAKTPLGELRFR
LcAE   MNFNVSLMEKLKWKIKCIENKFLNYRLTTNETVVAET.E....YGKVKGVKRLTVYDDSYYSFEGIPYAQPPVGELRFK
                                *          *    *              *   **       *      **


AcCE   DPV.PIEPWQEVREATEF.GP.MAAQFD.VISKFSGGSDDCLYINVYTKKINSNVK....QPVMFYIHGGGFIFG.SGN
MpCE   EPQ.PVQPWLGVWNATVPGSACLGIEFGS.GSKII.GQEDCLFLNVYTPKLPQENSAGDLMNVIVHIHGGGYYFGEG..
CqAE   DAQ.PPKPWTEPLDCTVQ.GP.GGYQYSKLLNKII.GREDSLHMNVFTKNLDSKQL....LPVMLYIHGGAFMRGSSGV
CtEB   PPV.PPPKWTETLDCTQQCEP..CYHFDRRIQKIV.GSEDSLKINVFAKEINPSNP....LPVLLYIYGGGFTEGTSGT
DmAE   APV.PADPWSGVLDCTHYAEK.P.TQRGLLTREIE.GGEDCLYLNVVSKQLKSEKP....LPVMVVIYGGAFTVGEATR
LcAE   APQRPT.PWDGVRDCCNHKDK.S.VQVDFITGKVC.GSEDCLYLSVYTNNLNPETK....RPVLVYIHGGGFIIGENHR
        *     *                              * * *  *          *  *  **       *


AcCE   DFFYGPDFLM.RKDIVLVTFNYRLGVFGFLNLEHEV..APGNQGLKDQVMALKWVRDNIANFGGDSENVTIFGESAGGA
MpCE   .ILYGPHYLLDNNDFVYVSINYRLGVLGFASTGDGVL..TGNNGLKDQVAALKWIQQNIVAFGGDPNSVTITGMSAGAS
CqAE   E.MYGPDYLI.QKDVVFVSFNYRIGALGFISFDSPELGLPGNAGLKDQNLALRWVVDNVANFGGDPKNITLFGESAGGC
CtEB   E.LYGPDFLI.QKDIVLVTFNYRIGALGFLCCQSEEDGVPGNAGLKDQNLAIRWVLENIAAFGGDPKRVTLAGHSAGAA
DmAE   E.LYGPDYFM.TKDVVLVTLNYRVDCLGFLSLKDPSLKVPGNAGLKDQVLALKWVKQYISNFGNDDSNITVFGESAGGC
LcAE   D.MYGPDYFI.KKDVVLINIQYRLGALGFLSLNSEDLNVPGNAGLKDQVMALRWIKNNCANFGGNPDNITVFGESAGAA
        ***         *  *     **    **        ** *****  *   *       * *     *  * ***

                                     ⇓
AcCE   SVHYLTVSPLAKGLFHKAISQSGVFMNPWASVSGEPRKKAYELCELLG.KKTTDPVEIVKFLRTVDTMKLIEHQGE.LQ
MpCE   SVHNHLISPMSKGLFNRAIIQSGSAFCHWSTAENVAQKTKY.IANLMGCP.TNNSVEIVECLRSRPAKAIAK...SYLN
CqAE   SVHYHMVSDLSRGLFQRAIVMSGCVLNNWSVV..PRRKFSERLAKALGWNGQGGERAALEVLVKADPESIVREQEVLLN
CtEB   SVQYHLISDASKDLFQRAIVMSGSTYSSWSLT..RQRNWVEKLAKTIGWDGEGGESGALRFLRAAKPEDIVANQEKLLT
DmAE   STHFMMCTEQTRGLFHKAIPMSGTVHNYWANN..PAEDFAFRLAQQNGFTGENDDAKVLEYLQGVPARDLV..NHNLLT
LcAE   STHYMMLTEQTRGLFHRGILMSGNAICPWANT..QCQHRAFTLAKLAGYKGEDNDKDVLEFLMKAKPQDLIKLEEKVLT
        *            **   *  **     *                    *            *            *


AcCE   IQELQKKCLSAFVPGVD....DKSPNPFMPFSREVAVEQA..AHVPYLIGYNDREGTLLY.KIFENDDFES.KNLRFE.
MpCE   FMPWRNFPFTPFGPTVEVAGYEK.FLPDIP..EKLVPHD.....IPVLISIAQDEGLIFSTFLGLENGFNELNNNWNE.
CqAE   ENEIENRILFSFGPVIEPYITKKCMIPKDPV..EMC.REAWSNEIDILIGGNSEEGLFCLNGIKENPSIMS..NL.KDF
CtEB   AEDMQEDIFTPFGPTVEPYLTEQCIIPKEPF..EMA.RNAWGDTIDVMIGGTSEEGLLLLQKVKLQPELLSHPHL....
DmAE   PEHRRNGLLFAFGPTVEAYVGEDCVVPKPPV..EMA.RDAWSNNFPVMLGGTSFEGLFMYPAVSAN..LKALDSLSQD.
LcAE   LEERTNKVMFPFGPTVEPYQTADCVLPKHPR..EMV.KTAWGNSIPTMMGNTSYEGLF.FTSI.....LKQMPMLVKEL
                  * *        * *                        **


AcCE   EFIH...P....NFAETLKRKKISLE...DLKRMYFKNKKI.SKETTGKFIDLFSDMYFIQGIHQV..ARVQAERNSAP
MpCE   .....HLP.HILDYNYTISNENLRFKTAQDIKEFYF.GDKPISKETKSNLSKMISDRSFGYGTSKA..AQHIAAKNTAP
CqAE   EYL...VPLEL....DLVRTSQRCKEVGKQMKKFYY.GETEPSFENREGYLTLMTDKLFLHGLHRTILSRLNS.KKPSK
CtEB   .FLGN.VPPHL....K.ISMEQRVAFAVK.LKQRYY.PDSDPSMENNSGYVHMMTDRVFWHGLHRTILARA.A.RSQAR
DmAE   PT..RLVP...VDV.RTVSSEKENLEYSQRLMKAYF.GYSPPSSELLLNMLDFYSYKIFWHGFNRTFNARLTY.AK.AP
LcAE   ETCVNFVPSELADAERTA...PETLEMGAKIKKAHVTG.ETPTAD...NFMDLCSHIYFWFPMHRLLQLRFNH.TSGTP
            *                                                                *

                                ▼
AcCE   TYMYQFTYDQGP..NFSKGMF...SIDEPGS..THMDELIYLFSMK.FQETLNM..EPIDKKSPHFRVMEQMVELWTNF
MpCE   VYFYEFGY.SG...NYSYVAFFDPKSYSRGSSPTHGDETSYVLKMDGFYVYDN.......EE..DRKMIKTMVNIWATF
CqAE   TFLYRFSVDS.DTYNHYRIVFCDKNV.R.GT..AHADDLSYIFK.......NVFNDPPAKDTFEHRAMMNMVGLFSTF
CtEB   TFVYRICLDS.EFYNHYRIMMIDPKL.R.GT..VHADELSYLFS.......NFTQQVPGKETFEFRGLQTLVDVFTSF
DmAE   TYYYRFDFDSPN.FNFYRAKFCGDKI.KTGV..AHADDLSYLFR.......NAGSWKLDKTSAEYRTIERMIGIWTAF
LcAE   VYLYRFDFDSEDLINPYRIMRSGRGV.K.GV..SHADELTYFFW.......NQLAKRMPKESREYKTIERMTGIWIQF
             *         *            *   * * *            *                        *


AcCE   AKY.GRP.IPAPTELLPVHWLPMNDG..T.VLRYLNIGEE.LRM......EK..VLNIEERYDY..KLICHREKV 532
MpCE   IK.SGVPD....TENSEI.WLPVSKNLADP.FRFTKITQQ..QTFEAREQSTTGIMNFGVAYH............ 552
CqAE   ASNNGNPNGEQINEWESI.ATPAG.....P.FKCLNINNDGLQFIEYPEQER..MKFWDSLYSK.DKLY...... 540
CtEB   VI.NGDPNCEMTSNSGVV.FEPNGQT..KPTFKCLNVSNEGVAFIDYPDPDR..LDMWDAMYVN.DELF...... 540
DmAE   AA.TSNPNCPEI.G..HLEWKPSTKN..DP.KRVINISSD.VTIIDLPEYEK..LQIWDNLY.KPNQLI...... 542
LcAE   AT.TGNPYSNEIEGMENVSWDPIKKS..DEVYKCLNISDE.LKMIDVPEMDK..IKQWESMFEKHRDLF...... 570
           *                    *
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Fig. 2. Predicted amino acid sequence of carboxylesterase-like enzyme from *A. calandrae* strain and alignment with esterases from other five insect species. ***AcCE***=carboxylesterase-like enzyme (GenBank: AF064523) of susceptible *A. calandrae*; ***MpCE***=carboxylesterase (GenBank: 544256) of *M. persicae*; ***CqAE***=A2 esterase (GenBank: 1905779) of *C. quinquefasciatus*; ***CtEB***=esterase B3 (GenBank: 1872538) of *C. tarsalis*; ***DmAE***=alpha esterase (GenBank: 1272308) of *D. melanogaster*; and ***LcAE***=esterase E3 (GenBank: 1336080) of *L. cuprina*. Functionally important residues are indicated by bold letters and solid triangles (▼) on the top of sequences. Identical residues among all six sequences are indicated with stars (★) at the bottom of sequences. Dots (.....) represent sequence alignment gaps. Conserved tryptophan (Trp^{220} in *A. calandrae*) residues are indicated by ⇓ on the top of sequences.

1997) did not suggest any signal peptide to be present. Esterase E3 from *Lucilia cuprina* lacked a signal peptide (Newcomb et al., 1997a), which is apparently a common feature in alpha-esterases (Robin et al., 1996).

The predicted carboxylesterase-like pro-enzymes from malathion-susceptible and resistant parasitoid cDNAs have molecular masses of 60,983 and 60,853 Da, respectively. These calculated molecular masses are very similar to that of MCE in *A. calandrae* determined by SDS–PAGE (Fabrick et al., unpublished). Depending on electrophoretic conditions, molecular mass of the MCE in *A. calandrae* is estimated to be between 55 and 60 kDa.

Theoretical p*I* values of 6.23 were obtained for deduced sequences of carboxylesterases from both parasitoid strains. The theoretical value is more basic than the p*I* of 5.2 for the MCE in *A. calandrae* determined by isoelectric focusing (Baker et al., 1998). We have evidence that the N terminus of MCE in *A. calandrae* is blocked (Fabrick et al., unpublished). Post-translational modification of proteins, particularly N-terminal blockage, can be responsible for differences between theoretical and experimentally derived p*I* values (Bjellqvist et al., 1994), and may be partly responsible for the difference found in *A. calandrae*.

3.3. Sequence difference between A. calandrae strains

Alignment of *A. calandrae* carboxylesterase-like enzyme cDNAs revealed that there were two nucleotide differences between the susceptible and resistant strains. At position 658 (Fig. 1), a thymine in the clone from the susceptible strain is substituted with a guanine in the clone from the resistant strain. This nucleotide change results in an amino acid difference in the predicted protein sequence from tryptophan (Trp^{220}) in the susceptible strain to a glycine (Gly^{220}) in the resistant strain. The point mutation at position 658 was confirmed in the genomic DNA sequence by using PCR amplification of specific allele (PASA) to amplify genomic DNA extracted from individual resistant and susceptible parents, F$_1$ hybrids from double reciprocal crosses, and backcross progeny (Zhu et al., 1999). The allele for glycine cosegregates with resistance and is inherited in a Mendelian fashion in both diploid female and haploid male progeny. Wasps in a mixed population of *A. calandrae* that survived a malathion screen carried the allele for glycine, while those wasps that died did not have the allele for glycine. At position 1122 (Fig. 1), an adenine in the susceptible strain is substituted by a guanine in the resistant strain. This nucleotide difference does not lead to a change in the predicted amino acid sequences. Codons ACA and ACG both code for threonine.

3.4. Comparison of carboxylesterase-like enzymes

A search of the GenBank database revealed that the sequence of the *A. calandrae* protein is similar to those of serine esterases from other insect species that are involved in organophosphate resistance through either a structural modification or amplification (Table 1). The *A. calandrae* enzyme is most similar to carboxylesterase from the peach-potato aphid, *Myzus persicae* (Sulzer), with 45% sequence identity (Field et al., 1993). The *A. calandrae* enzyme is also similar to esterase A2 from the mosquito, *Culex quinquefasciatus* (Vaughan et al., 1997), esterase B3 from the mosquito *C. tarsalis* (Tittiger and Walker, 1997), an alpha esterase in the fruit fly *Drosophila melanogaster* (Robin et al., 1996), and esterase E3 in the sheep blowfly, *L. cuprina* (Newcomb et al., 1996; Newcomb et al., 1997a).

The predicted amino acid sequence encoded by the susceptible *A. calandrae* cDNA was aligned with the five homologous insect esterases (Fig. 2). This sequence contains three active site residues, Ser^{187}, Glu^{317}, and His^{432}, corresponding to catalytic triad residues Ser^{200}, Glu^{327}, and His^{440} in acetylcholinesterase (AChE) of *Torpedo californica* (Sussman et al., 1991), which are conserved in all of these insect esterases (Field et al., 1993). Trp^{220} in susceptible *A. calandrae*, corresponding to Trp^{233} in AChE of *T. californica* (Sussman et al., 1991), is conserved when aligned with ten AChE from the Protein Data Bank and the five insect esterase sequences.

The three-dimensional structure of AChE from *T. californica* revealed that Trp^{233} is 4.3 Å from the γ oxygen of the catalytic Ser^{200}, which is centrally located in the esterase molecule. In the malathion-resistant strain of sheep blowfly *L. cuprina*, conserved Trp^{251} (=Trp^{233} in AChE of *T. californica*) is substituted by Leu^{251}, increasing the distance between the residue leucine and the catalytic serine from 4.3 Å to 6.0 Å (Campbell et al., 1998). This distance between Gly^{220} and the catalytic serine in the malathion-resistant stain of *A. calandrae* is expected to be even further. Campbell et al. (1998) hypothesize that the amino acid substitution of conserved tryptophan with leucine in *L. cuprina* creates more space around the catalytic serine which would reduce steric hinderance of the inversion about the phosphorus atom that must occur when the serine-phosphorus bond is hydrolysed. They also hypothesize that this might enable the relative position of the phosphorylated serine and catalytic histidine residues to change and bring the tetrahedral phosphate into a position that is more sterically favorable for hydrolysis of the serine-phosphate bond.

3.5. Carboxylesterase-like gene expression in two A. calandrae strains

Northern blots containing mRNAs and total RNA from susceptible and resistant *A. calandrae* were

Table 1
Similarity comparison and evolutionary relationship of an *A. calandrae* carboxylesterase-like enzyme to esterases from five other insect species

Species	Esterase	Similarity (%)	Identity (%)	Evolutionary distance
A. calandrae	carboxylesterase	–	–	
M. persicae	carboxylesterase	55	45	
C. quinquefasciatus	A2 esterase	56	44	
C. tarsalis	esterase B3	53	42	
D. melanogaster	α esterase	53	42	
L. cuprina	esterase E3	52	41	

hybridized with a carboxylesterase-like enzyme cDNA probe. Data indicated that the carboxylesterase-like enzyme cDNA probe hybridized to mRNAs and total RNAs of ~2 kb (Fig. 3A), which corresponds in size to the cloned carboxylesterase-like protein cDNAs. Northern blot analysis showed that expression levels of the carboxylesterase-like enzyme mRNA and total RNA in adults are significantly higher in resistant parasitoids than susceptible parasitoids. The same membrane was re-hybridized with a ribosomal protein S3 cDNA probe from *M. sexta* as an internal standard and the results showed that expression levels of S3 ribosomal protein are similar in the two strains (Fig. 3B). Densitometric analysis of the autoradiograph showed that carboxylesterase-like enzyme mRNA expressions analyzed using mRNA and total RNA were 28 and 32-fold higher (after calibration against loading control), respectively, in the resistant strain than in the susceptible strain.

3.6. Southern blot analysis of genomic DNA

Southern analysis was performed on genomic DNA from susceptible and resistant *A. calandrae* (Fig. 4). The

Fig. 4. Southern analysis and comparison of genomic DNA from malathion-susceptible strain (lanes 1–4) and resistant strain (lanes 5–8) of *A. calandrae*. S=susceptible and R=resistant. Five μg/lane of double-digested DNA was separated on 0.8% agarose gel; enzymes used for lanes 1 and 5=*Pst* I+*EcoR* I, lanes 2 and 6=*EcoR* I+*Hind* III, lanes 3 and 7=*Hind* III+*Pst* I, lanes 4 and 8=undigested control; M=DNA marker from BioRad. Digested DNA was transferred to nylon and hybridized with a full-length carboxylesterase-like cDNA labeled with ^{32}P-dCTP.
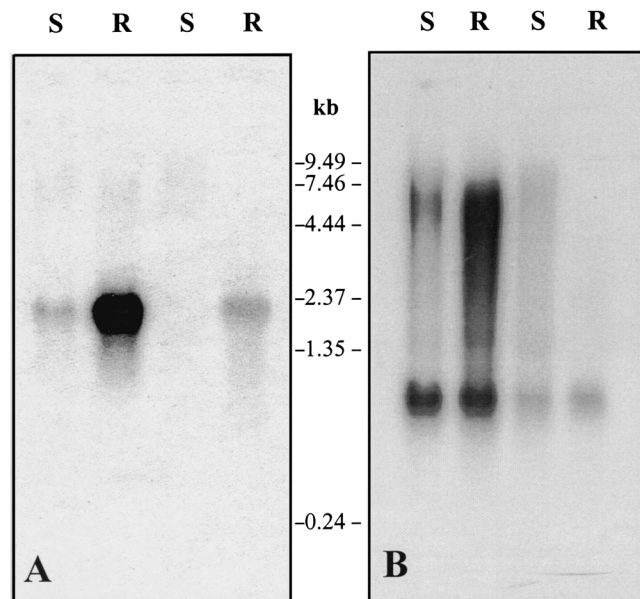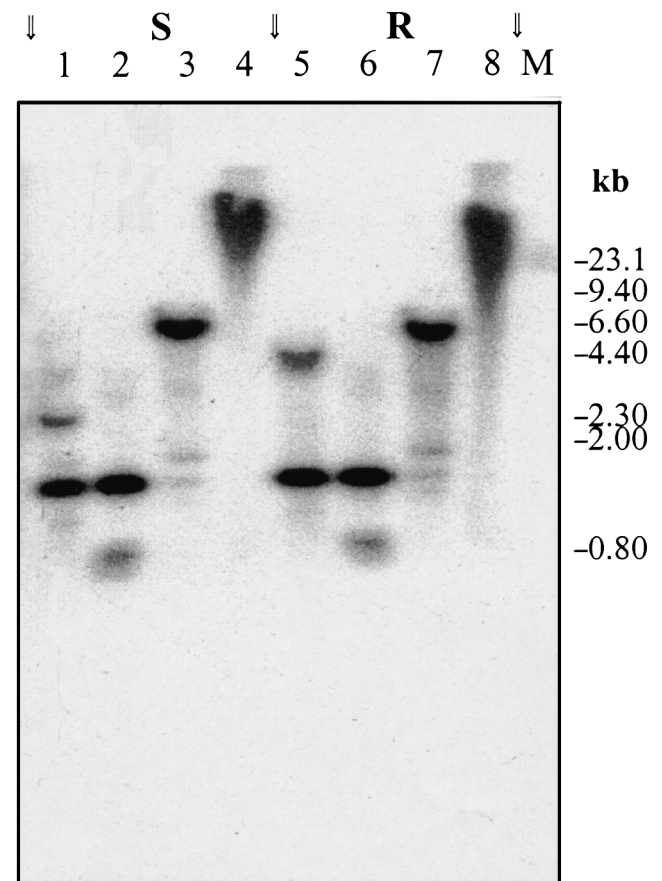
Fig. 3. Comparison of carboxylesterase-like mRNA expression levels in two *A. calandrae* strains. S=malathion-susceptible strain; R=malathion-resistant strain. (A) 3 μg of mRNA (lanes 1–2) and 10 μg total RNA (lanes 3–4) per lane from each strain was hybridized with a carboxylesterase-like cDNA probe labeled with ^{32}P-dCTP. (B) same nylon membrane from panel A, hybridized with a ribosomal protein S3 cDNA probe as a loading control.

carboxylesterase-like enzyme cDNA sequences from the susceptible and resistant strains of *A. calandrae* contain one *Hind III* (Position 1569) and two *EcoR I* restriction sites (Position 180 and 1167). No *Pst I* restriction site is found on the cDNAs. The Southern blot revealed two fragments (~1.4, ~2.5 kb) in the susceptible strain and two fragments (~1.4, ~4.4 kb) in the resistant strain in *Pst I+EcoR I* digests. The carboxylesterase-like enzyme cDNA probe hybridized to two fragments (~0.8 and ~1.4 kb) in both susceptible and resistant strain genomic DNAs produced by digestions with *EcoR I+Hind III* restriction enzymes. *Hind III+Pst I* digests showed one major hybridized band (~6.6 kb) and two smaller, less intense bands in both susceptible and resistant strain DNA (Fig. 4). These findings indicated that *Pst I* or *EcoR I* restriction sites were different between the susceptible and resistant strains. These restriction sites are probably located either on introns of the carboxylesterase-like enzyme gene or on untranscribed fragment regions.

3.7. Role of structural modifications and expression levels in resistant A. calandrae

Esterases are often found in multigene families (Oakeshott et al., 1993; Robin et al., 1996) and it is very likely that a complex of esterases is present in *A. calandrae*. During cDNA library screening in the current study, specific primers were designed according to the partial sequence of the carboxylesterase-like cDNA clone that was first isolated from *A. calandrae*. These primers were the only ones used to isolate carboxylesterase-like cDNA clones obtained from ^{32}P-labeled probe hybridization. These specific primers annealed only to highly similar carboxylesterase-like cDNAs. There may be other less similar esterase cDNAs present, and many other esterase genes may exist in the *A. calandrae* genome. The fact that we obtained what appear to be the relevant esterase cDNAs in the initial library screening may be a result of the specific primers, but it may also indicate that this gene has an abundant message, even in the susceptible strain. This would be consistent with the co-migration of MCE activity with the major α-naphthol acetate hydrolyzing activity in this species (Baker et al., 1998; P. Campbell, pers. comm.).

The structural modification Trp^{220}→Gly^{220} in resistant *A. calandrae* bears a striking similarity to one structural modification found in resistant blowflies. Esterase E3 in resistant *L. cuprina* differs from the susceptible allele in having five amino acid replacement sites (Newcomb et al., 1997b). The substitution Trp^{251}→Leu^{251} in E3 is most relevant to malathion resistance in this species (Campbell et al., 1998). An expressed allele with this substitution has a 10-fold lower *Km* for malathion, but has increased OP hydrolyzing activity compared with negligible OP activity in the susceptible allele. They

hypothesize that this small increase in OP hydrolyzing activity, rather than any kinetic differences in carboxylesterase activity against malathion, is primarily responsible for the high malathion resistance levels in this dipteran (ca. 600-fold). The enhanced OP activity is thought to be responsible for reactivation of the enzyme after phosphorylation by malaoxon during the intoxication pathway, i.e. the E3-Leu^{251} allele is less sensitive to inhibition by malaoxon than is the E3-Trp^{251} allele. In support of this hypothesis, Campbell et al. (1998) have shown that E3 variants in susceptible strains are very sensitive to inhibition by malaoxon and lack the ability to reactivate. This model relating structural modifications to malathion resistance is thought to be similar for at least 3 dipteran species (Campbell et al., 1997).

We do not know if the Dipteran model regarding effects of structural modifications will be applicable to the hymenopteran *A. calandrae*. Trp^{220} in *A. calandrae* (=Trp^{251} in *L. cuprina*) is highly conserved and the Trp^{220}→Gly^{220} substitution may result in an allele less sensitive to inhibition by malaoxon or with some other relevant biochemical property. However, expressed proteins of the wildtype and mutant forms are needed to confirm their MCE activity, and for additional kinetic studies, before we can attribute any specific biochemical function to the structural modification. We do know that a 10 to 30-fold increase of MCE activity in the resistant strain does not seem to be sufficient by itself to account for the >2500-fold malathion resistance documented in this parasitoid, a situation similar to that found in resistant *L. cuprina* (Campbell et al., 1998).

In addition to the structural mutation in the carboxylesterase from resistant *A. calandrae*, mRNA expression of a carboxylesterase-like enzyme is significantly higher in the resistant strain compared with that in the susceptible strain. Increased expression levels in the resistant strain may result from differences in gene regulatory elements or modification of regulatory factors for gene transcription. Insecticide resistance is often associated with either an increased expression of a detoxifying enzyme or a structural modification of the enzyme or target site. Our evidence suggests that both a structural modification and an increased expression of carboxylesterase-like enzyme may be involved in malathion-resistance in this parasitoid.

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